**APPLICATION FOR CONSENT TO RELEASE A GMO – ORGANISMS OTHER THAN HIGHER PLANTS**

**PART A1: INFORMATION REQUIRED UNDER SCHEDULE 2 OF THE GENETICALY MODIFIED ORGANISMS (DELIBERATE RELEASE) REGULATIONS 2002**

**19/DR/001/W**

**Part I**

**General information**

**1. The name and address of the applicant and the name, qualifications and experience of the scientist and of every other person who will be responsible for planning and carrying out the release of the organisms and for the supervision, monitoring and safety of the release.**

**Name and address of Applicant:**

Prokarium Ltd, London Biotechnology Innovation Centre, 2 Royal College St, London NW1 0NH.

**2. The title of the project.**

A Phase I, randomised, double-blind, placebo-controlled, parallel group dose escalation study to evaluate the safety, tolerability and immunogenicity of three doses of a potential oral enteric fever vaccine (ZH9 + ZH9PA) in healthy participants 18 to 45 years of age inclusive.

**Part II**

**Information relating to the Organisms**

**Characteristics of the donor, parental and recipient organisms**

**3. Scientific name and taxonomy.**

**Parental/Recipient organisms**

In this application the recipient organism is a genetically attenuated derivative of *Salmonella enterica* subspecies *enterica* serovar Typhi strain Ty2 (abbreviated to *S.* Typhi Ty2), which contains deletions in the *aroC* and *ssaV* genes as previously described in Application 10_R40_01 (15 Dec 2010). The attenuated derivative is known as *S.* Typhi (Ty2 *aroC*$^{-}$ *ssaV*$^{-}$) ZH9, abbreviated to ZH9. This strain was developed by Emergent Biosolutions as a single dose typhoid vaccine. Subsequently Emergent ceased this development and the technology was acquired by Prokarium Ltd., who are now developing the GMO in the present application.

There are two "donor" organisms involved in the construction of the novel GMO:

1. *Escherischia coli* strain O157:H7 which provides the sequence for the introduced *wbdR* gene. The donor material is a chemically synthesised plasmid containing the gene encoding an acetylase.

2. *Salmonella enterica* subspecies *enterica* serovar Paratyphi A which provides the sequence for the modified flagellin component *fliC*. The donor material is a chemically synthesised plasmid containing the gene encoding the H:a determinant.

The donor and recipient organisms are both bacteria.

The complete name of the recipient is as below:

| | |
|---|---|
| Kingdom: | Bacteria |
| Phylum: | Proteobacteria |
| Class: | Gamma proteobacteria |
| Order: | Enterobacteriales |
| Family: | Enterobacteriaceae |
| Genus: | *Salmonella* |
| Species: | *enterica* |
| Subspecies: | *enterica* |
| Serovar/Serotype: | Typhi |
| Strain: | Ty2 |

Genetic derivative: ZH9 (*aroC*$^{-}$, *ssaV*$^{-}$)

Attenuation of *S.* Typhi Ty2, to generate the recipient strain, ZH9, was achieved through the introduction of deletion mutations in two genes in the *S.* Typhi Ty2 chromosome. The first attenuating deletion is in the *aroC* gene which encodes chorismate synthase, an enzyme required for the biosynthesis of aromatic compounds. The safety of this attenuating strategy has been established in several clinical studies, this mutation being the basis of attenuation for several oral typhoid vaccines now in Phase II studies in the US. The second attenuating deletion is in the *ssaV* gene which is one of the genes of *Salmonella* pathogenicity island 2 (SPI-2), the genes of which encode a type III secretion system. The *ssaV* encodes a structural component of SPI-2, without which the type III secretion system is non-functional. *Salmonella* pathogenicity island 2 function is required for survival and growth of the bacterial cell in the host. The *ssaV* deletion mutation therefore prevents replication and systemic spread of the vaccine strain in humans. A mutation in *ssaV* provides an additional level of safety over that of the *aroC* mutation alone.

The *S.* Typhi Ty2 strain was originally isolated from an individual with typhoid fever in 1916 and has been used to derive all licensed parenteral and oral typhoid vaccines, including Vivotif, the current licensed oral typhoid vaccine, which has now been administered to several million subjects and has an excellent safety record.

**The donor organism**

The DNA sequences introduced into the recipient strain (ZH9) to produce the novel GMO were linear segments generated by restriction digestion of plasmids generated by chemical synthesis therefore there is no donor organism, strictly speaking. The nucleotide sequence information used to construct the transforming moieties was derived from databases containing the information.

**4. Usual strain, cultivar or other name.**

**Recipient strain**: *S.* Typhi Ty2(*aroC*$^{-}$ *ssaV*$^{-}$) ZH9

**Donor**: gene constructs generated by chemical synthesis; no donor organism

**Novel GMO**: The ZH9PA strain is *S.* Typhi ZH9 modified as follows: 1) to replace the H:d allele of the flagellin component *fliC* with the H:a allele; 2) to delete the gene encoding *rfbE* which converts abequose to tyvelose; 3) to replace the *rfbE* gene with the *wbdR* gene (an acetylase) as a spacer to maintain the expression of full length LPS-O sidechains.

The experimental enteric fever vaccine is an equal mixture of the parental organism (ZH9) with the novel GMO (ZH9PA) formulated with excipients.

**5. Phenotypic and genetic markers.**

Sequencing of 16S RNA genes identifies the parent strain and the novel GMO as strains of *S. enterica* with 99% confidence. The parent strain *S.* Typhi ZH9 and the novel GMO ZH9PA can be identified by performing dot-blots of whole bacteria with O:9 antisera and O:2 antisera respectively.

The parent strain and the GMO contain a 134 kb pathogenicity island, SPI-7, on the chromosome. Within this is a 15 kb region unique to all *S.* Typhi strains. The presence of bacteria containing this unique region has previously been used to monitor the survival of *S.* Typhi strains following addition to environmental samples.

The parent strain and novel GMO can be distinguished phenotypically from wild-type *S*. Typhi strains by culturing on selective agar media as the *aroC* mutation results in a requirement for a supplement of aromatic compounds for growth and by using polymerase chain reaction (PCR) analysis to show the presence of the *aroC* and *ssaV* gene deletions. Both strains contain:

- an *aroC* gene deletion of 600 bp and an insertion of 6 bp at the site of the deletion; in wild-type *S*. Typhi strains this gene is 1086 bp.
- an *ssaV* gene deletion of 1909 bp and an insertion of 16 bp at the site of the deletion; in wild-type *S*. Typhi strains this gene is 2046 bp.

The novel GMO can be distinguished genotypically from the parent strain by the presence of the H:a allele of the *fliC* gene and the absence of the *rfbE* gene.

**6. The degree of relatedness between the donor and recipient or between parental organisms.**

The plasmid components were generated by chemical synthesis; the donor organism (in terms of sequence information only) was Salmonella enterica ssp enterica serovar Paratyphi A (for the *fliC* gene) and *E. coli* (for the *wbdR* gene). The degree of relatedness between *E. coli* and *S. enterica* is 96.5% similarity based on 16S rRNA (Fukushima *et al.* 2002), the two species having diverged from a common ancestor approximately 100 million years ago (McQuiston *et al.* 2008).

**7. The description of identification and detection techniques.**

The recipient strain and the GMO can be cultured in the laboratory using standard selective media (Xylose Lysine Desoxycholate (XLD), Desoxycholate Citrate Agar (Hynes) (DCA (Hynes)), Selenite-F) and non-selective media (Brain Heart Infusion (BHI) and LB-aro) as a detection method.

Two PCR–based assays have been developed: the first to simultaneously identify any S. Typhi strain (by the presence of PCR products) and the parental strain ZH9 and novel GMO ZH9PA (by the difference in PCR product sizes). Both assays can be carried out on single colonies taken from nutrient agar plates. All PCRs use MyTaq™HS Red Mix 2X (Bioline, UK) and the cycling conditions below:

| | | |
|---|---|---|
| Stage 1: | Initial denaturation | 2 min, 95 ˚C |
| Stage 2: | Step 1 Denaturation | 10 s, 95 ˚C |
| | Step 2 Annealing | 10 s, 55 ˚C |
| | Step 3 Extension | 30 s, 72 ˚C |

Repeat steps 1-3 for 35 cycles

Stage 4: hold at 4 ˚C

The strain identification PCR assay uses two PCR reactions with different pairs of primers: one pair flanking the *aroC* gene and one pair the *ssaV* gene. These genes are deleted in ZH9, allowing ZH9 and derivative strains to be identified by size using agarose gel electrophoresis. Table 1 lists the primers and PCR product sizes.

Table 1: Colony PCRs to distinguish wild-type *S.* Typhi from the recipient strain *S.* Typhi ZH9 or the novel GMO ZH9PA.

| Locus | Primer name | Primer sequence (5'-3') | Product size (kb) | |
|---|---|---|---|---|
| | | | ***S.* Typhi** | **ZH9/ZH9PA** |
| *ssaV* | ssaV4 | ATCCCCACGACTTCAGGAAG | 2.6 | 0.7 |
| | ssaV7 | CTTTCTGGCTCATCATGAGG | | |
| *aroC* | aroC-L | CGGCGGCGATGGCGTCTTTATG | 1.3 | 0.75 |
| | aroC-R | CAACGTGTCGGCGCCAATGATG | | |

Table 2: Colony PCRs to distinguish the recipient strain *S.* Typhi ZH9 from the novel GMO ZH9PA

| Locus | Primer name | Primer sequence (5'-3') | Product size (kb) | |
|---|---|---|---|---|
| | | | **ZH9** | **ZH9PA** |
| *rfbE* (LPS) | L3 | TGTTCCTGCCGGTATAACTG | None | 1.06 kb |
| | L5 | CTGGCCATAATGCTTGTAATACCGCA | | |
| *fliC* (Paratyphi) | F4 | TGATGTGAAGAGCGAAGCGGT | None | 2.29 kb |
| | F9 | CTATGTCAGAAGATGAATCATCGACAGATCG | | |
| *fliC* (Typhi) | F10 | GCCTACACCCCGAAAGAAACTG | 2.27 kb | None |
| | F9 | CTATGTCAGAAGATGAATCATCGACAGATCG | | |

**8. The sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques.**

When the recipient strain and the GMO are recovered on selective media, and then tested using 16S rRNA sequencing, the specificity for identification as an S. enterica strain is 99.5%.

The colony PCRs described in Item 7 can be used to specifically detect S. enterica strains (simultaneously identifying and distinguishing between wild type, the recipient and the GMO) in complex environmental samples following recovery on selective media.

**9. The description of the geographic distribution and of the natural habitat of the organisms including information on natural predators, prey, parasites and competitors, symbionts and hosts.**

S. Typhi causes typhoid fever, a human-restricted systemic infection. This disease remains an important public health issue in many developing countries, predominating in areas with poor sanitation and limited access to potable water which together aid its transmission and persistence in the human population. The disease is endemic throughout Africa, Asia, central and South America, the Middle East and a few Southern and eastern European countries. Typhoid fever occurs sporadically in the developed world as a disease of travellers returning from endemic regions.

Human carriers are the main reservoir driving the transmission of S. Typhi, and the bacterium has a limited survival time outside the human host (Holt et al. 2008). Dispersal of wild-type S. Typhi occurs via faecal-oral transmission (contamination of food or water with faeces of infected individuals). A small minority (2-5%) of untreated individuals become chronic carriers and can shed S. Typhi in faeces for many years after the initial infection. In contrast, due to the presence of the attenuating mutations, the GMO is unable to colonise the human host for more than a few days. No evidence of prolonged shedding or chronic carriage, based on monitoring of faecal shedding, has been observed following administration of the recipient strain (ZH9) to 356 healthy subjects in six clinical studies in which it was monitored (see Item 32).

**10. The organisms with which transfer of genetic material is known to occur under natural conditions.**

Available evidence indicates that the propensity of S. Typhi for genetic exchange with other organisms is extremely low as there is very little genetic variation within the global S. Typhi population. Indeed, analysis of the whole genomes of 19 S. Typhi strains, selected to represent major nodes in the phylogenic tree, identified only 1,954 single nucleotide polymorphisms (SNPs) between all of them. Further, very little evidence of recombination between S. Typhi isolates or with other bacteria was found. Genomic insertions were rare in the sequenced isolates and evolution in the S. Typhi population seems to be characterised by ongoing loss of gene function caused by nonsense SNPs. These data support the hypothesis that evolution in S. Typhi is dominated by genetic drift and loss of gene function rather than by diversifying selection or gain of function through point mutation, recombination or acquisition of new sequences (Holt et al., 2008).

The S. Typhi ZH9 strain does not possess genes which would enable transfer of genes or plasmids to other bacteria by conjugation.

**11. Verification of the genetic stability of the organisms and factors affecting that stability.**

As discussed in Item 10, the genetic stability of S. Typhi is well characterised. Analysis of genomes from 19 S. Typhi strains revealed the highly monomorphic nature of the organism, with very little genetic variation in the global population. Genomic insertions were rare in the sequenced isolates and evolution in the S. Typhi population seems to be characterised by loss of gene function caused by nonsense SNPs (Holt et al., 2008).

**12. The following pathological, ecological and physiological traits:**

**a. the classification of hazard according to existing Community rules concerning the protection of human health and the environment;**

S. Typhi Ty2, the grand-parental strain of the GMO, is an ACDP classified Hazard Group 3 organism (Advisory Committee on Dangerous Pathogens: The Approved List of Biological Agents. Third edition. HSE 2013). Hazard Group 3 organisms are defined by COSHH as those that can cause severe human disease and may be a serious hazard to employees (Control of Substances Hazardous to Health Regulations 2002). S. Typhi Ty2 is classified as Biosafety Level 2 by the American Type Culture Collection (ATCC).

Work with the recipient strain (ZH9) and the novel GMO (ZH9PA) has been classified as a GM class 1 activity (of no or negligible risk) under the Genetically Modified Organisms (Contained Use) Regulations 2000 (as amended) (see Item 33). This classification is justified based on the 7 clinical trials in which S. Typhi ZH9 has been shown to well-tolerated and to have a good safety profile. In contrast to wild-type S. Typhi, and due to the presence of the attenuating mutations, neither the parent strain or the novel GMO is unable to colonise the human host. No evidence of prolonged shedding or chronic carriage, based on monitoring of faecal shedding, has been observed following administration of ZH9 and ZH9-derived strains to humans.

**b. the generation time in natural ecosystems, and the sexual and asexual reproductive cycle;**

S. Typhi divides asexually by binary fission and is host-restricted to humans. When cultured in vitro under conditions designed to support efficient replication it has a generation time of approximately 40 minutes. In contrast, S. Typhi does not survive for long periods in the environment. In simulated environmental conditions, no evidence of replication of the GMO was observed (see Item 55).

**c. information on survivability, including seasonability and the ability to form survival structures, including seeds, spores and sclerotia;**

In contrast with other enteric pathogens such as *Vibrio cholerae*, S. Typhi does not survive for long periods in the environment. S. Typhi does not form endospores or any other survival structures.

Emergent has studied the survival of the recipient organism (ZH9) and a less attenuated (aroC-) S. Typhi strain supplemented with aromatic compounds to overcome the attenuation in untreated sewage and the bacteria did not survive for longer than 21 days (see Item 55).

Survival of wild type S. Typhi in distilled water and 0.9% (w/v) sodium chloride has been studied by others. S. Typhi survived for 5 days in both at 37°C, 25 days in distilled water and 29 days in 0.9% (w/v) sodium chloride at 18-24°C and 65 days in both at 4-6°C when inoculated at 5 x $10^5$ CFU $mL^{-1}$ (Uyanik et al., 2008).

**d. pathogenicity, including infectivity, toxigenicity, virulence, allergenicity, carrier (vector) of pathogen, possible vectors, host range including non-target organisms and possible activation of latent viruses (proviruses) and ability to colonise other organisms;**

Humans are the only host for S. Typhi. It is transmitted from person-to-person by the faecal–oral route through infected food or water and gives rise to a systemic infection, typhoid fever. A bacterial concentration greater than 1000 colony forming units (CFU) is required to cause infection in healthy adults, and median incubation times and clinical attack rates have been shown to be dose dependent (Levine et al., 2001).

Following ingestion, bacteria rapidly invade the intestinal mucosa through the M cells or enterocytes to the Peyer's patches. From there bacilli are thought to be carried intra-cellularly to other reticulo-endothelial organs within 24 hours of ingestion. Entry into systemic circulation is from the mesenteric lymph nodes. Once in the blood, S. Typhi reside in macrophages. Following an incubation period of 7-14 days, bacteria can enter the blood stream causing a secondary bacteraemia, heralding the onset of clinical disease. Fever is thought to occur when the bacteria die and release endotoxin into the bloodstream. The type and severity of clinical symptoms varies, but can include fever, flu-like symptoms and headache along with the risk of serious secondary complications that may include intestinal haemorrhage, intestinal perforation, pneumonia and encephalopathy.

In the absence of treatment, bacteria can reach the gall bladder and induce an active local infection (cholecystitis) or exist asymptomatically in a chronic carrier state. Up to 5% of untreated patients suffering typhoid fever become chronic carriers (Crawford et al., 2008). Chronic carriage is defined as excretion of S. Typhi in stool for more than one year after clinical or sub-clinical infection. Chronic carriers are responsible for contributing to the "steady-state" of high infection rates in endemic areas.

In contrast, due to the presence of the attenuating mutations, the parent organism and the novel GMO are unable to infect and colonise the human host. Administration of the parent organism to 389 healthy subjects in seven clinical studies has shown the parent organism to be well tolerated and to have a good safety profile (refer to Item 32 for details of clinical studies).

**e. antibiotic resistance, and potential use of these antibiotics in humans and domestic organisms for prophylaxis and therapy;**

The parent strain and the novel GMO do not possess any antibiotic resistance genes.

**f. involvement in environmental processes including primary production, nutrient turnover, decomposition of organic matter and respiration.**

Not applicable due to the inability of the GMO strain to persist in the environment.

**13. The sequence, frequency of mobilisation and specificity of indigenous vectors and the presence in those vectors of genes which confer resistance to environmental stresses.**

No indigenous vectors are present.

**14. The history of previous genetic modifications.**

As described in Item 3.

**Characteristics of the vector**

**15. The nature and source of the vector.**

The modifications to the parent organism to create the novel GMO are all chromosomal, hence there is no vector DNA present.

**16. The sequence of transposons, vectors and other non-coding genetic segments used to construct the genetically modified organisms and to make the introduced vector and insert function in those organisms.**

The recipient strain contains a 6 bp restriction endonuclease recognition sequence inserted at the site of the *aroC* deletion. The recipient strain contains a 16 bp sequence, made up of two 6 bp restriction endonuclease recognition sequences separated by a 3 bp stop codon and one additional base pair, inserted at the site of the *ssaV* deletion. These elements have no functional significance. The recipient strain ZH9PA additionally contains a sequence of 58 bp adjacent to the inserted *wbdR* and deleted *fliC* genes which comprises the 28 bp *dif* (deletion-induced filamentation) sequence and restriction sites used to clone this in to the insertion/deletion plasmids. The *dif* site is required for the function of the 'Xer-cise' mechanism, as they are the recognition sites of the XerCD recombinase in *Salmonella* and originally flanked a chloramphenicol resistance gene (*cat*). Following chromosomal insertion under antibiotic selection, *cat* is excised by Xer recombination, generating a single *dif* site (Bloor and Cranenburgh 2006, Appl. Environ. Microbiol. 72: 2520–2525).

**17. The frequency of mobilisation, genetic transfer capabilities and/or methods of determination of the inserted vector.**

The novel GMO strain does not contain any vector-derived genetic material. Elimination of the vector sequences used in the generation of the recipient strain ZH9 has been confirmed by PCR and DNA sequencing (see Items 20(a), 24). The S. Typhi ZH9 and ZH9PA strains do not possess genes which would enable transfer of genes to other bacteria by conjugation.

**18. The degree to which the vector is limited to the DNA required to perform the intended function.**

N/A there is no vector

**Characteristics of the modified organisms**

**19. The methods used for the modification.**

Standard genetic manipulation methods were used throughout construction of the GMO (restriction enzyme digestion, DNA amplification by using polymerase chain reaction (PCR), DNA ligations, purification of plasmid DNA, bacterial transformation, electroporation, colony selection and identification, nucleotide sequence analysis; see Item 20 for further detail).

**20. The methods used –**

This content is unavailable due to it being confidential and commercially sensitive, as it has not yet been disclosed in a published patent

**21. The description of any insert and/or vector construction.**

See item 20.

**22. The purity of the insert from any unknown sequence and information on the degree to which the inserted sequence is limited to the DNA required to perform the intended function.**

The sequences of the modified loci have been determined and comprise only elements required for them to be functional or for the substitution of loci to be performed (i.e. the dif sequences and restriction endonuclease recognition sites). There are no unknown sequences present.

**23. The methods and criteria used for selection**

Chloramphenicol selection was used initially to confirm the transformation of ZH9 with the modified loci. The chloramphenicol resistance gene in the replacement cassettes is flanked by *dif* sequences, the recognition sites for Xer recombinases XerC and XerD found naturally in *Salmonella*. Subsequent culturing in the absence of chloramphenicol resulted in the excision of the chloramphenicol resistance gene and the formation of a single *dif* site (Bloor & Cranenburgh 2006). The resulting locus was fully sequenced to confirm 100% identity to the intended substitution.

**24. The sequence, functional identity and location of the altered, inserted or deleted nucleic acid segments in question and, in particular, any known harmful sequence.**

This content is unavailable due to it being confidential and commercially sensitive, as it has not yet been disclosed in a published patent.

**Characteristics of the genetically modified organisms in their final form**

**25. The description of genetic traits or phenotypic characteristics and in particular any new traits and characteristics which may be expressed or no longer expressed.**

The novel GMO differs from the parent strain only in the expression of modified LPS and *fliC* loci.

**26. The structure and amount of any vector or donor nucleic acid remaining in the final construction of the modified organisms.**

The GMO contains no vector DNA. The sequences of the loci modified in the GMO are aligned with the recipient strain loci in Item 24.

**27. The stability of the organism in terms of genetic traits.**

The genetic stability of ZH9 (the recipient organism) was previously studied well beyond the number of generations employed in the manufacturing process. For this study ZH9 was grown overnight on BHI agar plates supplemented with additional aromatic compounds. In order to avoid clonal selection ten single colonies were then picked and resuspended in liquid growth media. This pool

was streaked onto solid media and grown into single colonies. This process was the repeated until ten passages (rounds of growth) had occurred. Each colony picked is assumed to have arisen from a single bacterial cell, and for a bacterial colony to be macroscopic the total number of cells in the colony must be approximately 1 x $10^9$. This would require the single cell to undergo approximately 30 generations ($2^{30} \approx 10^9$) to form a visible colony. As ZH9 was passaged 10 times it therefore underwent approximately 300 generations. In order to confirm the stability of the mutations during passaging cells taken from the first and last agar plates were tested for the presence of the *aroC* and *ssaV* deletion mutations by PCR. The phenotype conferred by the *aroC* deletion mutation was also tested by comparing the growth on M9 minimal agar with casamino acids and the same media supplemented with additional aromatic compounds. These tests confirmed that both of the deletion mutations remained stable following growth through approximately 300 generations.

Genetic stability of the chromosomal modifications has therefore been demonstrated for a considerable number of generations beyond the release of the product. This would give a considerable safety margin if there were any replication within the vaccine recipient or the environment. This is considered to be highly unlikely as detailed in other parts of the application because of the way that ZH9 has been attenuated and by the fact that wild type *S.* Typhi does not naturally persist in the environment.

**28. The rate and level of expression of the new genetic material in the organisms and the method and sensitivity of measurement of that rate and level.**

The expression of the modified LPS and flagellin are similar in the novel GMO to their counterparts in the recipient strain. The levels of expression are evaluated in a number of ways, including immunostaining and FACS, and western blot analysis for both LPS and flagellin.

**29. The activity of the gene product.**

This content is unavailable due to it being confidential and commercially sensitive, as it has not yet been disclosed in a published patent.

**30. The description of identification and detection techniques, including techniques for the identification and detection of the inserted sequence and vector.**

As with the recipient strain ZH9 (see Item 7) the GMO can be cultured in the laboratory using standard selective media (XLD, DCA (Hynes), Selenite-F) and non-selective media (BHI). To culture the GMO, these media are routinely supplemented with aromatic compounds to complement the *aroC* mutation present in the GMO.

Colonies that have a morphology characteristic of S. Typhi are then sub-cultured for further identification using dot blots with antiserum against H:a flagellin and PCR reactions as described in item 7.

**31. The sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques.**

The biochemical and genetic analyses described in Item 30 allow the GMO to be identified with absolute specificity. The combination of analyses means that it is not possible to confuse identification of another micro-organism with the GMO.

The studies described in Items 8 and 55 show that it is possible to selectively culture and identify the GMO in a complex sample containing high concentrations of other organisms (> $10^6$ CFU $ml^{-1}$), such as untreated sewage, with complete specificity for, and differentiation of, isolated wild-type S. Typhi, ZH9 and ZH9PA by colony PCR.

**32. The history of previous releases or uses of the organisms.**

The GMO has not previously been released, however the recipient strain (ZH9) has previously been evaluated as a typhoid vaccine in seven clinical studies conducted in the UK, the United States and Vietnam. These studies are presented here as they are considered directly relevant to the GMO as the substitution of the LPS O chain and flagellin component FliC would not be expected to negatively impact either the safety profile of the organism or its ability to survive in the environment.

To-date the recipient strain (ZH9) has been administered to 389 healthy adult and paediatric participants up to a maximum dose level of 1.7 x $10^{10}$ CFU. Faecal shedding of the ZH9, the route of entry into the environment, was investigated in all but the last of these studies with various sampling frequencies and durations of follow-up to ensure the faecal shedding profile is well-understood. The data from the 6 trials in which shedding was evaluated are summarised in Table 3. In the most recent study, carried out under release authorisation 10_R40_01 (15 Dec 2010) at the University of Oxford, 33 participants were vaccinated with ZH9 but faecal shedding of the strain was not evaluated. More details of all of these studies is provided in the Investigators Brochure, provided as an appendix.

**Table 3. Number and percentage of participants with a stool sample positive for *S.* Typhi following dosing with the recipient strain, in each clinical study at each timepoint**

| | | | Time point post-dosing (days) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Study** | **Dose range (CFU)** | **Subjects** | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
| MS01.01<br>UK adults | 1 x $10^7$<br>– 1 x $10^9$ | 9 | 2<br>22.2% | 1<br>11.1% | 1<br>11.1% | NEG | NEG | NEG | 1<br>11.1% |
| MS01.03<br>US adults | 5 x $10^7$<br>– 5 x $10^9$ | 48 | 18<br>37.5% | 16<br>33.3% | 10<br>20.8% | 2<br>4.2% | 1<br>2.1% | 1<br>2.1% | NEG |
| MS01.04<br>US adults | 5 x $10^9$ | 32 | - | - | - | - | - | - | NEG |
| MS01.07<br>Vietnam adults | 5 x $10^9$ | 16 | - | 2<br>12.5% | - | - | - | - | NEG |
| MS01.08<br>Vietnam children | 5 x $10^9$ | 101 | 47<br>46.5% | 12<br>11.9% | 1<br>1.0% | NEG | NEG | NEG | NEG |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| MS01.13 US adults | $5 \times 10^9$ – $1.7 \times 10^{10}$ | 150 | - | 21/39* 53.8% | - | - | - | - | 3/150 2.0% |

| | Time point post-dosing (days) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Study** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **17** | **19** | **21** | **28** | **3 mths** | **6 mths** |
| MS01.01 UK adults | - | - | - | - | - | - | NEG | - | - | NEG | NEG | - | - |
| MS01.03 US adults | - | - | NEG | - | NEG | - | NEG | - | - | NEG | NEG | NEG | NEG |
| MS01.04 US adults | - | - | NEG | - | - | - | - | - | - | - | - | - | - |
| MS01.07 Vietnam adults | NEG | NEG | NEG | NEG | NEG | NEG | NEG | - | - | - | - | - | NEG |
| MS01.08 Vietnam children | NEG | NEG | NEG | NEG | NEG | NEG | NEG | - | - | - | - | - | - |
| MS01.13 US adults | - | 3/150 2.0% | - | 3/150 2.0% | - | - | 1/150 0.7% | 2/39* 5.1% | NEG * | NEG * | - | - | - |

*- = Not a scheduled stool sampling time point in the study,* NEG *= All faecal samples tested negative for S. Typhi, * = Only 1.7 x $10^{10}$ CFU cohort assessed at this time point*

The stool assay is comprised of a direct plating step and an enrichment step. A pea-sized piece of stool is streaked directly onto a DCAaro (Hynes) agar plate and also inoculated directly into selenite-F enrichment broth supplemented with aromatic compounds. Following overnight incubation, the selenite-F broth is used to inoculate a single DCAaro (Hynes) agar plate. Suspect S. Typhi colonies identified on the selective plates are subcultured on BHI agar plates supplemented with aromatic compounds. Positive identification of S. Typhi is confirmed by agglutination with specific antisera (O9, Vi, H(d)) and biochemical profiling with an API 20E test kit (BioMérieux). This assay has been qualified and the sensitivity determined to be 1.1 CFU S. Typhi 100/mg stool. In studies MS01.08 and MS01.13 the assay was used as described (the direct plating step omitted for MS01.13). In earlier studies (MS01.01, MS01.03, MS01.04 and MS01.07) very similar methodology was used and the level of sensitivity is expected to be similar to that of the qualified assay; for each of those studies the ability of the assay to detect S. Typhi was confirmed before study start.

The recipient strain has previously been released following administration to 9 healthy adult participants in a UK clinical study (MS01.01), 230 healthy adult participants in three clinical studies in the United States (studies MS01.03, MS01.04, MS01.13), and 16 healthy adults (study MS01.07) and 101 healthy children in Vietnam (study MS01.08). Most recently the recipient strain was released

following administration to 33 healthy adult participants in a UK clinical study (OVG 2011/02) approved under Release 10-R40-01. A summary of each clinical study, including details of monitoring for safety and for release of the GMO via shedding, is provided in the Investigators Brochure.

**33. In relation to human health, animal health and plant health -**

The GMO contains two independently attenuating deletions in *ssaV* and *aroC*. In accordance with the Genetically Modified Organisms (Contained Use) Regulations 2000 (as amended), a risk assessment has been carried out and work with the GMO was classified as a GM class 1 activity (of no or negligible risk).

There are no anticipated effects upon either plant or animal health as, in addition to the highly attenuated nature of the GMO, S. Typhi is a tightly host-restricted pathogen of humans. Therefore, human health only is considered in the sections below.

**a. the toxic or allergenic effects of the non-viable organisms and/or their metabolic products,**

The recipient strain has previously been administered to 389 participants in seven phase 1 and 2 clinical studies (see Item 32) and no toxic or allergenic effects were reported.

The GMO has been evaluated in formal toxicology studies in small animal models with no adverse findings.

**b. the comparison of the organisms to the donor, recipient or (where appropriate) parental organism regarding pathogenicity,**

The recipient organism (ZH9) has been assessed in human participants at a range of doses up to 1.7 x $10^{10}$ CFU (see Item 32 for a summary of all clinical studies performed to date). In all studies safety monitoring was a critical component and included monitoring the vaccinees closely for any signs or symptoms of infection related to the attenuated S. Typhi vaccine strain. Participants were monitored for the presence of the recipient organism in blood and urine at defined study visits or as clinically indicated (dependent upon the study protocol). Stool samples were cultured regularly (with different sampling frequencies and duration of follow-up according to the study protocol) to establish the shedding profile in faeces. As described in more detail in Item 32, shedding of the recipient organism following administration is transient as the recipient organism is unable to colonise vaccine recipients, and faecal shedding only occurs as a consequence of it passing through the gastrointestinal tract following ingestion. This is in contrast to infection with wild-type S. Typhi, where chronic carriage of the bacterium can occur in a small minority (2-5%) of untreated individuals with faecal shedding ongoing for many years (see Item 32).

The GMO is expected to exhibit the same lack of pathogenicity as the parent organism as no additional virulence determinants have been introduced. There has been a simple like for like exchange of LPS O-chains and Flagellin alleles.

**c. the capacity of the organisms for colonization, and**

Wild type S. Typhi strains are severely host restricted, only having the capacity to colonize humans. The recipient strain is severely attenuated and unable to colonize humans as highlighted by the lack of bacteraemia and chronic carriage in any clinical study participants (see Item 32). The GMO is expected to have the same inability to colonise as the recipient strain as the flagellin and LPS changes made confer no properties which will change this phenotype.

**d. if the organisms are pathogenic to humans who are immunocompetent -**

Neither the recipient strain nor the GMO are pathogenic to humans. The *aroC* and *ssaV* mutations incorporated into S. Typhi Ty2 are specifically designed to attenuate the ability of the GMO to cause disease and no evidence of invasiveness (i.e. bacteraemia, chronic carriage and/or long-term faecal shedding) was observed in any of the 356 healthy participants that have been administered the GMO and monitored for shedding to date. Moreover, the GMO is not expected to be pathogenic in immuno-compromised individuals. *Salmonella aro* mutants have been shown to be attenuated in immuno-compromised animal models. Additionally, *ssaV* mutants are unable to replicate in macrophages, and it has been shown that SPI-2 mutants are unable to kill severely immuno-compromised animals. Pre-clinical studies have shown that the recipient strain is unable to replicate in a human macrophage-like cell line (Khan et al., 2007), and a study using an immunocompromised IFN-γ mouse model of infection has shown that an *aroC- ssaV-* double mutant was completely safe in this model. This indicates that the attenuation of the GMO is not dependent on the ability of the innate or specific immune system to control the replication or spread of the organism and it should therefore be attenuated in immuno-compromised individuals.

**i. diseases caused and mechanisms of pathogenicity including invasiveness and virulence,**

The GMO is non-pathogenic

**ii. communicability,**

There is no evidence of person-to-person transmission of ZH9.

**iii. infective dose,**

N/A as the GMO will not establish an infection.

**iv. host range and possibility of alteration,**

S. Typhi is exquisitely adapted to humans and is unable to infect any other species.

**v. possibility of survival outside of human host,**

The GMO is highly attenuated for survival in the environment.

**vi. presence of vectors or means of dissemination,**

There are no vectors present.

**vii. biological stability,**

The GMO is highly stable after many cycles of cell division grown in optimal culture conditions.

**viii. antibiotic-resistance patterns,**

The GMO is susceptible to common antibiotics and carries no known antibiotic resistance determinants.

**ix. allergenicity, and**

None known.

**x. availability of appropriate therapies.**

N/A.

**e. Other product hazards**

No hazards are anticipated to result from release of the GMO due to the severe attenuation of this strain.

**Part III**

**Information relating to the conditions of release**

**The release**

**34. The description of the proposed deliberate release, including the initial purpose or purposes of the release and any intention to use the genetically modified organisms as or in a product in the future.**

The GMO will be administered to healthy participants in a Phase I placebo-controlled clinical study. The clinical protocol will be submitted to the Medicines and Healthcare products Regulatory Agency (MHRA) as part of a clinical trials application (CTA). The purpose of the study is to investigate the safety of and immune responses to ascending doses of the GMO, or placebo in healthy participants. In relation to the release of the GMO, the main points are summarised below.

Participants will be recruited at a single clinical study site:

**Simbec-Orion Clinical Pharmacology, Simbec House, Merthyr Tydfil Industrial Park, Pentrebach, Merthyr Tydfil, Mid Glamorgan, CF48 4DR, United Kingdom**

A maximum of 45 participants will be included in the study and randomised to one of three treatment groups, to receive the novel GMO (ZH9PA) at two different dose levels, placebo or a mixture of the ZH9PA and ZH9. The GMO will be diluted in sodium bicarbonate solution, with the possible addition of ascorbic acid and aspartame, for oral administration. The highest dose level of ZH9PA and ZH9 given to each participant will be $1 \times 10^{10}$ CFU.

In previous clinical studies it has been shown that after administration, the recipient strain may be transiently shed in the faeces of participants for up to 17 days after dosing (see Item 32).

Shedding in faeces thus constitutes the release of the GMO and consequently the GMO may be released into the sewage system. In relation to release into the sewage system, Emergent Biosolutions has previously generated data that show that the recipient strain does not survive in untreated sewage (see Item 55); Prokarium believes that the alterations made to the LPS and flagellin loci will not have any impact on the survival of the GMO. In addition, stools from patients suffering with typhoid fever, following infection with a wild-type strain of S. Typhi, are discharged into the sewage system and they are effectively contained by normal sewage treatment processes; the UK sewage system has significantly contributed to the eradication of enteric diseases such as typhoid and cholera from the population. When the GMO enters the sewage system it has the potential to come into contact with the wider environment (e.g. soil and water bodies). All available data indicates that the vaccine strain is unable to persist in these environments (see Item 55).

Due to the extensive data on shedding of the recipient strain (and of related strains of S. Typhi) collected in previous clinical trials, it is considered that the shedding profile of the GMO is now sufficiently well-characterised (see Item 32). It is therefore not proposed to monitor stool samples taken from participants in the study for the presence of the GMO itself at any point other than at the

end of the study to demonstrate that no persistent shedding is occurring and no subject has become a carrier. Other monitoring procedures will be carried out in the study as described in Item 76.

To minimise accidental transmission of the GMO to surfaces or to other individuals (non-target hosts), the participants will be instructed to maintain strict personal hygiene during the study and proper hand washing techniques will be taught and reinforced. Faecal-oral transmission of the GMO from the participants to other people is a consideration for this study and strict exclusion criteria have been set to minimise such transmission, in particular to minimise the risk of transmission to potentially vulnerable groups (see Item 68).

The GMO may form the basis of a vaccine product in the future.

**35. The intended dates of the release and time planning of the experiment including frequency and duration of releases.**

The clinical study is expected to begin in September 2019. The first participant is expected to be dosed shortly after the study start date; recruitment may take up to 24 months; the last dose of GMO to be administered to a participant will therefore occur before September 2021. The GMO will be given orally to participants who are likely to shed the organism in faeces at low levels; shedding has not been seen for longer than 17 days in previous studies with the recipient strain but higher doses will be used in this study and shedding may go on for longer in a small number of participants. It is not anticipated that shedding will continue beyond 3 weeks. This shedding constitutes the release of the GMO, thus it is anticipated that the release will end within 21 days of the last dose being administered to the last participant (September 2021). The duration of the release (dosing/shedding phase of the study) is expected to be no longer than 25 months.

For details of the dosing schedule for participants in each of the treatment groups, refer to the study synopsis, provided as an appendix.

**36. The preparation of the site before the release.**

The clinical study site, at which the GMO will be administered to participants, will be initiated according to GCP and according to documented local procedures prior to study start. This will include training of the investigator(s) and all other members of the proposed study team, as appropriate.

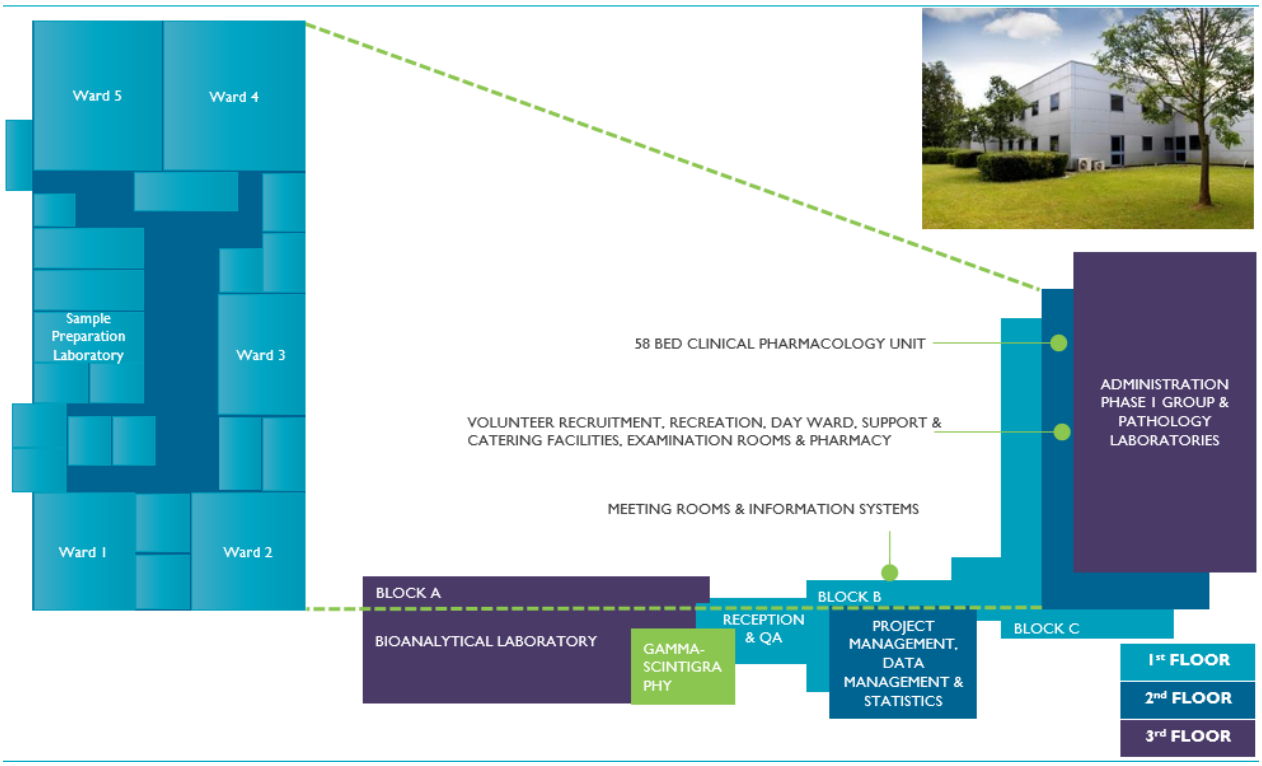
**37. The size of the site.**

The proposed site of release is the Clinical Pharmacology Unit of Simbec-Orion Clinical Pharmacology.

This study will be conducted by Simbec-Orion's specialist Phase I unit, Simbec-Orion Clinical Pharmacology, based in South Wales, UK. Simbec-Orion Clinical Pharmacology is a full-service MHRA accredited facility with over 40 years' experience, the site includes a 58-bed clinical pharmacology unit and our dedicated in-house central pathology and bioanalytical laboratories; it also houses our volunteer recruitment unit, pharmacy and project management department.

The proposed site of release is the Clinical Pharmacology Unit based on the second floor of Simbec-Orion Clinical Pharmacology as shown in figure 6.

Figure 6: Plan of Simbec-Orion Clinical Pharmacology Research Facility




**38. The method or methods to be used for the release.**

Study participants will be randomised to receive the GMO, with or without the parent strain ZH9, or placebo. Participants will receive the GMO by oral delivery, by drinking the GMO diluted in sodium bicarbonate buffer solution as described below.

The GMO is supplied as a frozen suspension of cells which is diluted in the bicarbonate buffer. The dose must be given within 30 minutes of preparation. Participants will be instructed to fast for ninety minutes before and after dosing. Participants will be observed for a period of four hours after dosing before being allowed to go home.

The GMO solution for ingestion will be prepared at the trial site, according to detailed dosing instructions provided by the Sponsor.

Participants will receive three doses of the GMO, at 21-day intervals.

Following dosing, the GMO may be shed from participants in faeces into the sewage system. A stool sample will be taken at D70 (28 days after the last administration of the GMO) to confirm that the participants are no longer shedding. In the unlikely event that a positive stool culture is obtained, then those participants will be asked to return to the clinical unit to receive a course of antibiotics,

previously shown to be effective at eradicating ZH9. On Day 84, participants will be asked to provide a second stool sample to confirm that shedding has ceased.

**39. The quantity of organisms to be released.**

A maximum of 45 participants will be included in the study. Of these, a maximum of 30 participants will be dosed with the GMO. The maximum release of the GMO ZH9PA in the study overall will be no more than $2.5 \times 10^{11}$ CFU. The maximum release of the parent ZH9 in the study overall will be no more than $1.2 \times 10^{11}$ CFU.

**40. The disturbance of the site, including the type and method of cultivation, mining, irrigation, or other activities.**

Not applicable.

**41. The worker protection measures taken during the release.**

All clinical staff will be appropriately trained according to GCP and documented local procedures, including the SOP's of Simbec-Orion. Trial personnel in direct contact with participants and/or responsible for handling the GMO will use personal protective equipment (aprons and gloves) as appropriate. Other Infection Control Policies relevant to worker protection, covering e.g. disposal and decontamination procedures, and procedures for the management of accidental spillage, are described in Items 42, 77, 79, 82 and 84.

**42. The post-release treatment of the site.**

Following each release event (i.e. each administration of a dose of GMO), the dosing area will be cleaned and disinfected.

In clinical areas, routine cleaning of surfaces will be performed according to Simbec-Orion SOPs using alcohol wipes. Larger spillages will be treated with chlorine-releasing tablets 1.7g (Actichlor) in situ for 2 minutes, then cleared up, wearing appropriate personal protective equipment. The area will then be washed with hot water and detergent. In laboratory areas, surfaces (including floors and benches) will be decontaminated with 1% Virkon solution. Minor surface contamination will be treated with 1% Virkon for 10 minutes. Larger spillages (including blood and body fluids) will be treated with Virkon granules for a minimum of 10 minutes.

Any equipment used for dosing will be cleaned and decontaminated (e.g. by autoclaving) or disposed of (e.g. by incineration), as appropriate. All disposable materials will be contained as appropriate in clearly labelled bags or sharps bins and disposed of as clinical waste according to Simbec-Orion SOP's. All disinfection, decontamination and disposal procedures will be performed wearing suitable personal protective equipment in accordance with documented local procedures including those for Infection Control. Disposal of waste generated during the release is described in Item 82.

**43. The techniques foreseen for elimination or inactivation of the organisms at the end of the experiment or other purposes of the release.**

As a consequence of release, the GMO may be released into the sewage system. Potentially contaminated faeces released into the public mains sewers will be treated according to standard sewage treatment procedures.

It is possible that some of the shed organisms could enter environmental niches other than the sewage system, e.g. soil and water bodies, if a breach of the sewage system were to occur or if faecal samples containing the GMO were disposed of via facilities that do not involve a mains sewage system. Studies performed to investigate the survivability of the parent strain (ZH9) in soil and aqueous environments showed that S. Typhi carrying the attenuating mutations does not persist, surviving for a limited time only (see Item 55).

**44. Information on, and the results of, previous releases of the organisms and in particular, releases on a different scale or into different ecosystems.**

This is the first time that ZH9PA will be released, however the recipient strain (ZH9) has previously been administered to 389 healthy adult and paediatric participants in seven clinical studies to assess safety and immunogenicity (see Item 32).

**The environment (both on the site and in the wider environment)**

**45. The geographical location and national grid reference of the site or sites onto which the release will be made, or the foreseen areas of use of the product.**

The address of the proposed site of release is as follows:

Simbec-Orion Clinical Pharmacology, Simbec House, Merthyr Tydfil Industrial Park, Pentrebach, Merthyr Tydfil, Mid Glamorgan, CF48 4DR, United Kingdom

The national (OS) grid reference of the proposed site of release is SO 064033.

**46. The physical or biological proximity of the site to humans and other significant biota.**

The site is in Pentrebach, Merthyr Tydfil, which is an urban commercial area.

**47. The proximity to significant biotopes, protected areas or drinking water supplies.**

The site is approximately 200 m from the River Taff.

**48. The climatic characteristics of the region or regions likely to be affected.**

N/A

**49. The geographical, geological and pedological characteristics.**

N/A

**50. The flora and fauna, including crops, livestock and migratory species.**

Not applicable. The organism is restricted in its host range to humans.

**51. The description of target and non-target ecosystems likely to be affected.**

As a consequence of release, the GMO may be released into the public sewage treatment system.

**52. The comparison of the natural habitat of the recipient organisms with the proposed site or sites of release.**

The natural habitat of the GMO and the parent strain is humans. Humans will be the only recipients of the GMO. As a consequence of release, the GMO may be released into the public sewage treatment system and there is a possibility that the GMO could enter other environmental niches e.g. soil and water bodies, if a breach of the sewage system were to occur or if faecal samples containing the GMO were disposed of via facilities that do not involve a mains sewage system.

**53. Any known planned developments or changes in land use in the region which could influence the environmental impact of the release.**

None known.

**Part IV**

**Information relating to the interactions between the organisms and the environment**

**Characteristics affecting survival, multiplication and dissemination**

**54. The biological features which affect survival, multiplication and dispersal.**

Wild type S. Typhi is host-restricted and only infects humans. It does not infect, and is not harboured by, any animals or plants. Human carriers are the main reservoir driving the transmission of S. Typhi via faecal contamination of food and water supplies, and it is generally accepted that the bacterium has a limited survival time outside the human host (Holt et al., 2008). S. Typhi is not capable of forming spores or generating other specialist structures to enhance environmental survival.

Wild type S. Typhi is released into the environment following replication in the human host. Following infection, S. Typhi replicates in macrophages of the liver and spleen, before being released and widely disseminated in the blood. Secondary infection of the gallbladder is followed by faecal shedding into the environment. Up to 5% of patients suffering typhoid fever become chronic carriers (Crawford et al., 2008). Chronic carriage, usually asymptomatic, is defined as excretion of S. Typhi in stool for more than one year after infection or sub-clinical infection. Chronic carriers are responsible for contributing to the "steady-state" of high infection rates in endemic areas.

The GMO contains two independently attenuating mutations in *aroC* and *ssaV* (see Item 24 for details). As a consequence of the attenuation, the vaccine strain is unable to cause infections and the duration of shedding is limited (see Section 33 b for comparison of the GMO and wild-type S. Typhi). Further details of the effects of the attenuating mutations on pathogenesis are provided in Items 12(d) and 24. Clinical data obtained following administration of the parent strain of the GMO (ZH9) to 389 participants in 7 clinical studies have shown no signs and symptoms of typhoid fever and no bacteraemias; ZH9 was shed in stool for a maximum of 17 days post-dosing (the majority of subjects (97.8%) did not shed S. Typhi for longer than 7 days) (see Item 32). Accordingly, entry of the GMO into the environment by faecal shedding is limited, and as shown in Item 55, the GMO is not capable of replication and survival in the environments into which it may pass.

**55. The known or predicted environmental conditions which may affect survival, multiplication and dissemination, including wind, water, soil, temperature and pH.**

It is expected that following release of GMO by faecal shedding, it will be contained within the sewage system and will then be exposed to the normal sewage treatment processes as is the case for wild-type S. Typhi. The stools from any patient suffering from typhoid fever disease are discharged into the sewage system and wild type S. Typhi within them is effectively contained by normal sewage treatment processes. The importance of sewage treatment is underlined by the fact that typhoid fever was an important cause of illness and death in the United States and Europe in the 19th century. The provision of clean water and good sewage systems led to a dramatic decrease in the incidence of typhoid in these regions (Parry et al., 2002).

It is acknowledged that GMOs entering the sewage system have the potential to come into contact with other environmental niches. In addition, it could be possible that the faecal samples containing the GMO may be disposed of via facilities that do not involve a mains sewage system and from here the GMO may enter other environmental niches. An extensive set of studies have therefore been conducted with the parent strain of the GMO (ZH9) to determine its capability to survive in the following environments:

- Untreated sewage
- River water
- Seawater
- Soil

The modifications made to ZH9 to generate the GMO provide no benefit to the survival of the GMO in the environment. Prokarium Ltd believes that the data generated with ZH9 are directly relevant to the GMO.

**Untreated sewage**

A colony blot hybridisation technique was developed that specifically detects S. Typhi strains in mixed bacterial populations. Using a DNA probe designed to specifically hybridise to a unique gene contained within SPI-7 on the S. Typhi chromosome, this approach allows the rapid screening of large numbers of bacterial colonies on agar plates to identify S. Typhi. Screening increased numbers of colonies at each time point increases the power of the method and lowers the limit of detection (LOD) for S. Typhi in the samples.

An untreated, screened (to remove solid debris) sewage sample was collected from the inlet works at Reading Sewage Treatment Works, Reading, UK. The bacterial microflora population of the sample was determined to be 2.24 x $10^6$ CFU/mL and single aliquots were inoculated with ZH9 or S. Typhi (Ty2 aroC-) DTY8 at a ratio of approximately 10:1 GMO:sewage flora. S. Typhi (Ty2 aroC-) DTY8 contains a single mutation in the aromatic pathway which can be complemented by the addition of aromatic compounds to growth media. It is used here as a control to mimic the survival properties of a “wild type” S. typhi strain, whilst being amenable to manipulate in the laboratory as a category 2 organism. (Fully wild-type S. typhi is category 3). The aliquot inoculated with DTY8 was supplemented with aromatic compounds to complement the *aroC* mutation of the strain, and therefore this strain acted as a “wild-type” S. Typhi comparator. A third aliquot was inoculated with PBS to confirm specificity of the colony blot hybridisation detection method.

All aliquots were held statically in the dark at 20°C, and the S. Typhi population of each was monitored by sampling on days 0, 1, 3, 7 and 21 following inoculation. At each timepoint bacteria were recovered on agar media, and the S. Typhi colonies detected by colony blot hybridisation using the S. Typhi-specific DNA probe described in Item 7. This blotting technique has been developed to specifically detect S. Typhi in mixed bacterial populations, allowing large numbers of colonies to be screened at each timepoint.

ZH9 was inoculated into the sewage sample at a concentration of 2.11 x $10^7$ CFU/mL. Following colony blot hybridisation analysis of the samples, the S. Typhi population was found to be 50.8% (2.14 x $10^6$ CFU/mL) of the total viable cell count (VCC) at day 0, it declined sharply to 2.6% (1.65 x $10^5$ CFU/mL) at day 1, and to 0.19% (83 CFU/mL) by day 7. S. Typhi ZH9 was not detected at the day 21 timepoint (LOD 1.5 CFU/mL).

A similar profile was observed for S. Typhi (Ty2 aroC-) DTY8. This strain was inoculated into the sewage sample at a concentration of 1.77 x $10^7$ CFU/mL, and colony blot analysis of the samples at day 0 showed the S. Typhi population to be 36.4% (9.61 x $10^5$ CFU/mL) of the total VCC. The S. Typhi population then declined sharply to 1.69% (2.82 x $10^5$ CFU/mL) at day 1 and 0.28% (1.21 x $10^3$ CFU/mL) by day 7. S. Typhi was not detected at the day 21 timepoint (LOD 5 CFU/mL).

S. Typhi was not detected at any time point in the assay control (sewage inoculated with PBS).

It was concluded from this study that neither the parent strain of the GMO (ZH9) nor the wild-type comparator (S. Typhi (Ty2 aroC-) DTY8 plus supplementary aromatic compounds) was able to persist in untreated sewage, when inoculated at a ratio of approximately 10: 1 GMO:sewage flora, for more than 21 days.

**River water**

A river water sample was collected from the River Thames in Caversham, Reading, UK, filter-sterilised and split into 7 aliquots. Triplicate aliquots were inoculated with ZH9 at 1.4 x $10^7$ CFU/mL (high inoculum) and at 1.4 x $10^5$ CFU/mL (low inoculum); a final river water aliquot was not inoculated and was used as a negative control.

All aliquots were then incubated statically in the dark at 25°C and analysed at various timepoints following inoculation. At each timepoint the viable cell count of the samples was determined on BHI agar plates supplemented with aromatic compounds. Samples were taken on day 0, 1, 3 and 6, and every 2 to 3 days thereafter. The study proceeded for each sample until no viable cells were recovered at two consecutive time points.

The S. Typhi population in all inoculated samples declined by approximately 1 log per 6 days. For the low inoculum sample ZH9 had declined to 1.4% of the starting inoculum level at day 6, to 0.5% at day 10 and to 0.005% at day 20. ZH9 was detected up to and including day 24 and was not detected on day 27 or 28. ZH9 therefore did not persist beyond day 27 in this sample. In the high inoculum sample ZH9 had declined to 6.6% of the starting inoculum level at day 6, to 2.5% at day 10 and to 0.01% at day 20. ZH9 was detected up to and including day 36 and was not detected on day 41 or 42. S. Typhi was not detected at any time point in the negative control river water sample. The LOD for the assay was 1.7 CFU/mL.

It was concluded from this study that the parent strain of the GMO (ZH9) did not persist in river water. ZH9 survived for no longer than 41 days in the high inoculum samples and for no longer than 27 days in the low inoculum samples. The rate of decline of the GMO in river water was similar for both inoculum levels tested.

**Seawater**

A seawater sample was collected from the coastline at Bournemouth Beach, Dorset, UK, filter-sterilised and split into 4 aliquots. Duplicate aliquots were inoculated with ZH9 at 2.65 x $10^7$ CFU/mL (high inoculum) and at 1.56 x $10^5$ CFU/mL (low inoculum). All aliquots were then incubated statically in the dark at 25°C, and samples taken daily to determine the total viable cell count on XLD agar plates supplemented with aromatic compounds. The study proceeded for each sample until no viable cells were recovered.

For the low inoculum sample ZH9 declined rapidly to 0.03% of the starting inoculum level at day 1, and on day 2 no viable cells were recovered. For the high inoculum sample ZH9 declined to 0.3% of the starting inoculum level at day 1, 0.003% on day 2 and by day 4 ZH9 could no longer be detected. The LOD for the assay was 3.3 CFU/mL.

It was concluded from this study that the parent strain of the GMO (ZH9) does not persist in seawater. ZH9 survived for no longer than 4 days in the high inoculum samples and for no longer than 2 days in the low inoculum samples.

These data are in line with those published by Wait and Sobsey (2001), where the population of S. Typhi ATCC 19430 inoculated into seawater was shown to take an average of 3.8 days to reduce by 90% at 20°C in the laboratory.

**Soil**

Commercially sourced topsoil was sterilised by heat treatment and stored in 1g aliquots. Aliquots were inoculated with ZH9 at concentrations of 1 x $10^4$, 1 x $10^5$, 1 x $10^6$ and 1 x $10^7$ CFU/g and incubated at room temperature (~ 22°C). The viable cell count of samples was determined by recovery onto BHI agar plates supplemented with aromatic compounds at the following timepoints: 1, 2, 3 and 6 days post-inoculation for the 2 lowest inoculum levels; 2, 4 and 8 days post-inoculation for the 1 x $10^6$ CFU/g samples and 1, 3 and 7 days post-inoculation for the 1 x $10^7$ CFU/g samples. Samples from each inoculum level were assessed until no viable cells were recovered.

It was concluded from this study that ZH9 survived for only a limited time in soil. ZH9 persisted for no longer than 8 days at the 2 highest inoculum levels tested and no longer than 6 days at the 2 lower inoculum levels.

**Other environmental conditions**

Regarding the impact of other environmental conditions on the survival of S. Typhi in the environment, a published study on survival in aqueous systems suggests that the duration of survival may be impacted by temperature. Uyanik et al. (2008) found that S. Typhi survived for 5 days in distilled water and 0.9% (w/v) sodium chloride at 37°C, 25 days in distilled water and 29 days in 0.9% (w/v) sodium chloride at room temperature, and 65 days in both solutions at 4-6°C.

In conclusion, it is generally accepted that S. Typhi has a limited survival time outside the human host and due to the nature of the attenuation of the GMO, it is expected that the vaccine strain

would persist for no longer than a wild-type strain surviving only transiently in the environment. Additionally, data have been generated in an extensive set of studies to show that the parent strain of the GMO (ZH9) does not persist in untreated sewage, river water, seawater or soil.

**56. The sensitivity to specific agents.**

The GMO is sensitive to the antibiotics normally used for antibiotic therapy for typhoid fever i.e. ciprofloxacin, ampicillin, and trimethoprim-sulphamethoxazole.

**Interactions with the environment**

**57. The predicted habitat of the organism.**

The GMO is predicted not to have any habitat as humans are the only host for wild type S. typhi and the GMO is non-pathogenic in man. The GMO will be present transiently in the gastrointestinal tract of vaccinees and will be shed in faeces into the environment for a short time, however the organism is unable to survive in the environment (see Item 55).

**58. The studies of the behaviour and characteristics of the organisms and their ecological impact carried out in simulated natural environments, such as microcosms, growth rooms and greenhouses.**

N/A

**59. The capability of post-release transfer of genetic material-**

**a. from the genetically modified organisms into organisms in affected ecosystems,**

The GMO contains no plasmids, so transfer of genetic material is considered to be extremely unlikely.

**b. from indigenous organisms to the genetically modified organisms.**

As a relatively recently evolved human pathogen, S. Typhi is highly monomorphic, meaning there is very little genetic variation within the global S. Typhi population, thereby indicating that its propensity for genetic exchange is extremely low.

This is supported by a study that analysed of the whole genomes of 19 S. Typhi strains and identified only 1,954 single nucleotide polymorphisms (SNPs) between all of them; approximately 1 every 2,300 bp. Further, very little evidence of recombination between S. Typhi isolates or with other bacteria was found. Genomic insertions were rare in the sequenced isolates and evolution in the S. Typhi population seems to be characterised by ongoing loss of gene function caused by nonsense SNPs. All data in this study supports the hypothesis that evolution in S. Typhi is dominated by genetic drift and loss of gene function rather than by diversifying selection or gain of function through point mutation, recombination or acquisition of new sequences (Holt et al., 2008).

The risk of the GMO acquiring copies of the deleted aroC and ssaV genes to regain a virulent phenotype, via genetic exchange (conjugation, transduction) with a donor organism, is considered to be negligible.

Firstly, the two deletions in the GMO, both of which independently attenuate the strain, are physically separated on the chromosome by 793 kb (based on S. Typhi Ty2 genome sequence – GenBank Accession Number AE014613.1). Therefore, while it is theoretically possible that both mutations could be complemented, this is extremely unlikely, as it would require two separate complementation events. In support of this assessment, S. Typhi strains carrying single mutations in aroC have been administered to humans in a number of clinical trials and reversion to virulence has never been reported (Tacket et al., 1992b; 2000b; 2004). Similarly, reversion of the parent strain of the GMO to virulence has never been observed (refer to Item 32). Furthermore, normal gut flora does not contain the ssaV gene.

**60. The likelihood of post-release selection leading to the expression of unexpected or undesirable traits in the genetically modified organisms.**

The likelihood of post-release selection leading to the expression of unexpected or undesirable traits in the vaccine strain is negligible. The GMO contains two independently attenuating mutations and is therefore at a competitive disadvantage.

**61. The measures employed to ensure and to verify genetic stability, the description of genetic traits which may prevent or minimise dispersal of genetic material and methods to verify genetic stability.**

As the deletions introduced attenuate the ability of the strain to replicate in the human host, the only natural reservoir of S. Typhi, this minimises the risk of genetic dispersal. The modified DNA would offer no survival advantage to organisms in the environment.

A description of the studies performed to show the genetic stability of the strain *in vitro* is included in Item 27.

Evidence of the genetic stability of the parent strain of the GMO (ZH9) in vivo was gained in the Phase II clinical studies MS01.08 and MS01.13 (see Item 32 for clinical study outlines). In both studies, robust monitoring of faecal shedding of ZH9 was conducted. All S. Typhi strains isolated from stool samples were retained and characterised by PCR. In all cases, isolates contained the *aroC* and *ssaV* chromosomal deletions, demonstrating that the deletion mutations are stable during passage of ZH9 through human participants.

**62. The routes of biological dispersal, known or potential modes of interaction with the disseminating agent, including inhalation, ingestion, surface contact and burrowing.**

Dispersal of wild-type S. Typhi occurs via faecal-oral transmission (contamination of food or water with faeces of infected individuals).

In this release, the GMO will be excreted directly into the sewage system and it is expected that it will be contained there to be subject to normal sewage processing treatments. It is expected, based on evaluation of shedding in previous clinical trials with the parent strain (ZH9), that the GMO will be shed by participants for no longer than 17 days post-dosing.

Strict exclusion criteria have been set for the trial to minimise the risk of transmission of the GMO, and in particular to minimise transmission to potentially vulnerable groups such as immuno-compromised individuals, pregnant women or the very young (refer to Item 68 for more details of the exclusion criteria). Participants will be instructed on how to maintain strict personal hygiene and proper hand washing will be taught and reinforced to minimise the risk of faecal-oral transmission.

**63. The description of ecosystems to which the organisms could be disseminated.**

In this release the GMO will be excreted into the sewage system and subjected to normal sewage treatment processes, which are considered suitable for the treatment of wild type S. Typhi (since individuals infected with typhoid fever are not, in the UK, required to take any special precautions in the disposal of their stools). There is also a possibility that the GMO could enter other environmental niches such as soil and water bodies, should a breach in the sewage system occur, or if faecal samples containing the GMO were disposed of via facilities that do not involve a mains sewage system.

**64. The potential for excessive population increase of the organisms in the environment.**

The potential for excessive population increase of the organisms in the environment is negligible. Wild-type S. Typhi is unable to persist in the environment and the GMO does not have a competitive advantage in relation to the wild-type strain. All studies undertaken with the vaccine strain indicate that it cannot survive in the environment (see Item 55).

**65. The competitive advantage of the organisms in relation to the unmodified recipient or parental organism or organisms.**

The GMO has been designed to have a strong disadvantage compared to wild-type S. Typhi, because it possesses two deletion mutations that result in the severe attenuation of the strain. The *aroC* mutation renders the GMO auxotrophic for p-aminobenzoic acid, 2,3-dihydroxybenzoic acid and aromatic amino acids that are not available in human tissue, and the *ssaV* mutation prevents replication and systemic spread.

**66. The identification and description of the target organisms if applicable.**

Target organisms are carefully selected human participants in the clinical trial.

**67. The anticipated mechanism and result of interaction between the released organisms and the target organisms if applicable.**

It is anticipated that following oral ingestion by the human participants the GMO will reach the small intestine and interact with the intestinal mucosa such that a host immune response is generated

against the GMO. It is anticipated that this immune response will be protective against wild-type S. Paratyphi A infection.

**68. The identification and description of non-target organisms which may be adversely affected by the release of the genetically modified organisms, and the anticipated mechanisms of any identified adverse reaction.**

Wild-type S. Typhi has a very narrow host range and cannot colonise any organisms other than humans.

A consideration for the GMO is faecal-oral transmission from the participants to non-target hosts. Strict exclusion criteria have been set for the trial including criteria to minimise the risk of transmission of the GMO, and in particular to minimise transmission to potentially vulnerable groups. The relevant exclusion criteria are:

- Working as a food handler, in child-care or as a healthcare worker with direct patient contact.
- Have household contacts who are <2 years old or >80 years old or infirm or immunocompromised (for reasons including corticosteroid therapy, HIV infection, cancer chemotherapy, or other chronic debilitating disease).
- Pregnancy, risk of pregnancy, or lactation (female subjects only).

All participants enrolled into the trial will also be instructed to maintain strict personal hygiene and proper hand washing will be taught and reinforced, to minimise the risk of faecal-oral transmission.

**69. The likelihood of post-release shifts in biological interactions or in the host range.**

The GMO is derived from wild type S. Typhi Ty2, which is severely host restricted, only having the capacity to colonise and cause disease in humans. The GMO is an attenuated derivative of S. Typhi Ty2, harbouring deletion mutations in two genes that are required for growth and replication within the host. It is highly unlikely that the introduction of these mutations will change the host range of the GMO, and indeed the attenuated organism could be described as having a reduced host range compared to the wild type as it is no longer able to colonise human participants. Evaluation of the GMO in toxicity and safety studies in mice has shown no evidence of a change in host range.

**70. The known or predicted interactions with non-target organisms in the environment, including competitors, prey, hosts, symbionts, predators, parasites and pathogens.**

None expected.

**71. The known or predicted involvement in biogeochemical processes.**

None expected.

**72. Any other potentially significant interactions with the environment.**

None expected.

**Part V**

**Information on monitoring, control, waste treatment and emergency response plans**

**Monitoring techniques**

**73. Methods for tracing the organisms and for monitoring their effects.**

Extensive assay development work has been performed to ensure that the GMO can be isolated by in vitro culture methods. Once cultured, positive identification of S. enterica can be confirmed with 16S rRNA sequencing. A multiplex PCR assay can then be applied as required, to distinguish the GMO from the parent strain ZH9 and wild-type S. Typhi (see Items 30 and 31).

The assay used to detect shedding of the GMO in the stool of vaccine recipients has been qualified and it was demonstrated that 1.1 CFU per 100 mg stool could be detected (see Item 32).

Using these sensitive assay methods, the shedding profile of the parent of the vaccine strain has been assessed in six phase I and II clinical studies, and it was established that shedding of ZH9 in faeces occurred for no longer than 17 days after administration (see Item 32).

A colony blot hybridisation technique has been developed that can specifically detect and quantify S. Typhi strains in complex samples such as untreated sewage without the need for laborious isolation techniques prior to identification (see Items 7, 8, 30, 31 and 55).

Regular check monitoring of public mains water by water supply companies is in place in the UK to monitor for potential environmental contamination, and they will respond as per regulations for coliform bacteria (The Water Supply (Water Quality) Regulations (2018). (http://www.legislation.gov.uk/wsi/2018/647/contents/made).

**74. Specificity (to identify the organisms and to distinguish them from the donor, recipient or, where appropriate, the parental organisms), sensitivity and reliability of the monitoring techniques.**

The methods described in Item 73 are completely selective for the identification of the S. Typhi strains. When used in conjunction with the described PCR, the detection methods are completely specific for the detection of the GMO and the parent strain ZH9 (see Item 30 for discussion).

**75. Techniques for detecting transfer of the donated genetic material to other organisms.**

The risk for genetic transfer with organisms is low; the reasons are outlined in Item 59. However, transfer of the donated genetic material could be identified by PCR (see Item 74).

**76. Duration and frequency of the monitoring.**

Participants in the clinical trial will have stool cultures performed at Day 70 (28 days after their last dose) to document the fact that they are no longer shedding the GMO. In the unlikely event that a positive stool culture is obtained, then those participants will be given a course of antibiotics, which

have shown effectiveness in eradicating ZH9. Following this course of antibiotics, participants will be asked to return for a repeat stool sample on Day 84 to confirm that shedding has ceased.

**Control of the release**

**77. Methods and procedures to avoid and/or minimise the spread of the organisms beyond the site of release or the designated area for use.**

**Methods and procedures at the site of release**

Personal protective equipment will be used as appropriate (laboratory coat, apron, safety glasses, and disposable gloves). Only authorised and trained staff will be permitted to enter the clinic rooms and laboratory. Access to the clinical facility is controlled by physical locks, magnetic locks with key fobs to open and intruder alarms.

The GMO will be administered in a designated room with separate hand washing facilities. Following dosing with the GMO, participants will stay at the Study Centre under observation for 4 hours. The waste from the sanitary facilities at the site enters directly into the public sewers which are capable of containing the organism.

After dosing, all surfaces will be disinfected according to documented local procedures (see Item 42). All clinical waste, including tissues, disposable clothing and other miscellaneous waste will be placed into biohazard bags contained within closed bins and disposed of as clinical waste according to Simbec-Orion SOP's (Item 82).

**Methods & procedures beyond the site of release**

The release of the GMO into the environment will effectively occur after participants have been dosed and have left the clinical site, when the vaccine strain is expected to be shed in stools for a period of up to 21 days.

The GMO will therefore enter the sewage system during this period. It has been shown that the vaccine strain does not survive in untreated sewage (see Item 55) and the sewer is engineered to contain waste prior to processing.

In view of the low infectivity of S. Typhi and the level of hygiene and sanitation in the UK, secondary transmission of the GMO to household contacts or other close contacts is considered highly unlikely. It is thought that S. Typhi is virtually never transmitted by direct faecal-oral contact, due in part to the requirement for ingestion of a high inoculum of S. Typhi bacteria in order to cause clinical disease.

Participants will be educated in hand-washing to avoid secondary transmission of S. Typhi, using the National Patient Safety Agency guidance on hand washing and will be given detailed advice on how to prevent transmission of S. typhi. They will also receive guidance on procedures for dealing with soiled clothing, bed linen etc., so as to minimise secondary transmission.

When occasional transmission of typhoid fever occurs, it is usually related to unknowingly infected food handlers. For this reason, as described in Item 68, food handlers are excluded from the proposed study. Potential participants employed in clinical or social work with direct contact with young children or vulnerable patients or persons in whom typhoid infection would have particularly serious consequences also represent an increased risk and will be excluded (unless willing not to work from the point of dosing until demonstrated not to be infected with S. Typhi, in accordance with guidance from the Health Protection Agency); refer to Item 68 for a full description of these exclusion criteria.

**78. Methods and procedures to protect the site from intrusion by unauthorised individuals.**

The doors of the clinical unit are only accessible to authorised personnel with Simbec-Orion Clinical Pharmacology key cards at all times. Activities at the facility are regularly monitored with 24/7 security cover. The GMO will be stored in securely locked freezers except for those periods when vaccine doses are being prepared and administered.

**79. Methods and procedures to prevent other organisms from entering the site.**

All staff working in the facility will use personal protective equipment (laboratory coat, apron, safety glasses, and disposable gloves) as appropriate and will follow local documented procedures for Infection Control, which should minimise the risk of other organisms entering the facility.

Good laboratory practice and aseptic technique will be followed as necessary. The facility is self-contained with its own equipment and is separate from other activities in the building.

**Waste treatment**

**80. Type of waste generated.**

- Laboratory waste (plastic ware, liquid reagents, residual GMO).
- Clinical waste (faecal, urine, blood samples; sharps).
- Miscellaneous waste (disposable clothing, tissues).

**81. Expected amount of waste.**

Small amounts of waste, which can be handled by standard procedures, will be generated.

**82. Description of treatment envisaged.**

All waste disposal and decontamination procedures will be performed using appropriate personal protective equipment, in accordance with local documented procedures for Infection Control. Where applicable, waste (e.g. plastic ware, liquid reagents, microbial cultures, blood, stool and urine samples) will be pre-disinfected by full immersion in 1% Virkon (or 2% Virkon if heavy contamination) for at least 2 hours. All waste, including pre-disinfected material, will be placed in biohazard bags and disposed of as clinical waste according to Simbec-Orion SOPs. Used sharps will be discarded straight into a sharps container at the point of use, prior to disposal as clinical waste.

**Emergency response plans**

**83. Methods and procedures for controlling the organisms in case of unexpected spread.**

If any of the participants vomit following administration of the GMO at the clinical site, this will be treated as a biological hazard. Suitable personal protective equipment and disinfectant will be used in the inactivation of the hazard. All resulting waste will be disposed of into sealed containers for autoclaving and incineration, in accordance with local documented procedures for waste disposal (Item 82) and for the management of patients with vomiting and diarrhoea. However, since the GMO is severely attenuated it will not survive outside the human host.

All study samples and specimen sample bags must be labelled with a 'Danger of Infection' label and transported in accordance with local documented procedures.

Wild-type S. Typhi is a human-specific pathogen with no animal, plant or insect vector. The GMO is a severely attenuated form of S. Typhi unable to infect or colonise healthy adults. In an emergency situation measures will be put in place to identify persons who are susceptible to infection with the GMO, may have become infected with, or are carriers of the GMO.

The GMO is sensitive to ciprofloxacin, an antibiotic that is licensed for human use in the event of infection with *Salmonella* sp. This antibiotic is effective in the treatment of acute infection and eliminating chronic carriage. In the case of children, alternative effective antibiotics (e.g. ampicillin, trimethoprim/sulphamethoxazole) are available.

Prophylactic antibiotics can also be used in exposed individuals before infection has been established.

Contaminated areas may be decontaminated by the use of standard disinfectants. The efficacy of the disinfectant can be tested by swabbing the disinfected area and inoculation into appropriate media.

Transmission of the organism within the environment is readily controlled by sewage treatment processes.

**84. Methods, such as eradication of the organisms, for decontamination of the areas affected.**

Contaminated areas may be decontaminated by the use of standard disinfectants (e.g. Virkon, 70% ethanol, FAM30).

The methods for decontamination of areas (surfaces, equipment) affected as a result of unexpected spread will be essentially the same as those described for decontamination of areas following each scheduled release event (please see Item 42).

**85. Methods for disposal or sanitation of plants, animals, soils and any other thing exposed during or after the spread.**

Treatments with disinfectants, incineration or autoclaving are all effective means for decontamination of exposed items.

**86. Methods for the isolation of the areas affected by the spread.**

Participants will be monitored for signs/symptoms of ill health throughout the study; should an illness occur that is felt to be related to infection with the GMO, the participant will be treated with antibiotics as considered appropriate (see Item 76).

**87. Plans for protecting human health and the environment in case of the occurrence of an undesirable effect.**

If any of the participants vomit following administration of the GMO, this will be treated as a biological hazard. Suitable personal protective equipment and disinfectant will be used to inactivate the hazard. All resulting waste will be disposed of into sealed containers for clinical waste, in accordance with local documented procedures for waste disposal (Item 82) and for the management of patients with vomiting and diarrhoea (Item 83).

The proposed clinical trial procedures involve close clinical monitoring of participants for any adverse events, should an illness occur that is felt to be related to infection with the GMO, the participant will be treated with antibiotics as considered appropriate.

In addition to the above information, a risk assessment has been carried out in accordance with the Genetically Modified Organisms (Contained Use) Regulations 2000 (as amended) and work with the GMO was classified as a GM class 1 activity (of no or negligible risk) (see **items 12(a) and 33**).

**Part VI**

**A description of the methods used or a reference to standardised or internationally recognised methods used to compile the information required by this schedule, and the name of the body or bodies responsible for carrying out the studies.**

The modification of ZH9 to produce the GMO, ZH9PA, results in a straightforward replacement of two surface antigens. The LPS O:9 serotypic determinant is replaced with LPS O:2 and the flagellin H:d serotypic determinant is replaced with H:a. These changes have no impact on the other microbiological properties of the GMO and so the methods used to characterise the parent strain, ZH9, and the results obtained are directly applicable to the GMO, ZH9PA.

The methods used to:

- Construct the GMO are described in **Item 19** and **20** of this application
- Determine genetic stability of the parent strain of the GMO are described in **Item 27**
- Determine persistence of the parent strain of the GMO in soil and different aquatic systems are described in **Item 55**
- Evaluate replication of the parent strain of the GMO in a macrophage-like cell line is described in Khan *et al.*, 2003
- Evaluate safety of the attenuating mutations in immunocompromised mice are widely published, standard procedures
- Assess safety of the GMO in mice were according to:
    - CPMP/SWP/2145/00 Note for guidance on non-clinical local tolerance testing of medicinal products.
    - - CPMP/SWP/465/95 Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines
    - - WHO/BS03, 1969 Guidelines on Non-clinical Evaluation of Vaccines.
    - - ICH S6(R1) (CHMP/ICH/731268/1998): Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals.
- Assess toxicity of the GMO in the mouse model were standard acute dose toxicity procedures
- Monitor faecal shedding in clinical trial subjects are described in **Item 32**

All the above studies on the parent strain of the GMO (ZH9) were performed by Emergent Product Development UK Limited, or by contract research companies with oversight by Emergent Product Development UK Limited.

All the above studies on the GMO (ZH9PA) were performed by Prokarium Limited, or by contract research companies with oversight by Prokarium Limited.

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**APPENDIX 1: Investigators' Brochure – enclosed as separate document**

**APPENDIX 2: Study Synopsis and Schedule of Events**

**APPENDIX 2: Study Synopsis and Schedule of Events (extracted from Protocol v2.0 (12 July 2019)**

# SYNOPSIS

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|---|
| **NAME OF COMPANY:** Prokarium Ltd. |
| **NAME OF INVESTIGATIONAL MEDICINAL PRODUCT:** ZH9PA and ZH9/ZH9PA |
| **NAME OF ACTIVE INGREDIENT:** ZH9 and ZH9PA |
| **TITLE OF STUDY**: A Phase I, randomised, double-blind, placebo-controlled, parallel group dose escalation study to evaluate the safety, tolerability and immunogenicity of three doses of a potential oral enteric fever vaccine (ZH9 + ZH9PA) in healthy participants 18 to 45 years of age inclusive. |
| **PRINCIPAL INVESTIGATOR:** Dr Annelize Koch |
| **STUDY CENTRE:** Simbec-Orion Clinical Pharmacology, Merthyr Tydfil, CF48 4DR, UK |
| **CLINICAL PHASE:** I |
| **OBJECTIVES:**<br><br>Primary Objective<br>• To evaluate the safety and tolerability of three doses of two dose levels of ZH9PA and of three doses of a single dose level of a combination of ZH9 and ZH9PA over 12 weeks (up to Day 84) after the first dose of vaccine.<br><br>Secondary Objectives<br>• To evaluate serum immunoglobulin (Ig) A and IgG responses against the following antigens: - *S. enterica* Ser. Paratyphi A lipopolysaccharide (LPS) 0:2 and Flagella H:a and *S. enterica* ser. Typhi LPS O:9 and Flagella H:d, induced by three vaccine doses, of two dose levels ($1x10^9$ colony forming units (CFU) and $1x10^{10}$ CFU) of ZH9PA alone or in combination with $1x10^{10}$ CFU ZH9, up to Day 84 after the first dose of vaccine (Day 0).<br><br>• To evaluate the mucosal IgA immune responses to LPS 0:2, LPS O:9, Flagella H:a and Flagella H:d, at 7 days after each vaccination, as determined by antibodies in lymphocyte supernatant (ALS) assay.<br><br>• To evaluate Serum IgA and IgG responses against the four antigens, induced by each of the first two vaccine doses, on Days 21, and 42, by blood samples taken prior to each participant receiving the next dose of vaccine or placebo.<br><br>• To evaluate the safety profile of the ZH9PA and combination ZH9/ZH9PA vaccines, over the 6 months following the third dose of vaccine. |
| **METHODOLOGY:**<br>This is a Phase I, randomised, double-blind, placebo-controlled, parallel group, single-centre study involving 45 healthy participants. The aim is to evaluate a combination vaccine against enteric fever comprising the live attenuated Salmonella strain ZH9 (previously tested in a number of clinical trials as a typhoid vaccine) and its modified derivative, ZH9PA, in which the typhoid antigens LPS O:9 and Flagellin H:d have been substituted by the Paratyphi A antigens, LPS O:2 and Flagellin H:a. ZH9PA has not previously been tested in humans; therefore, the first two cohorts comprise a dose escalation of this component alone, followed by a |

final cohort in which the combination is tested at a single dose level. The study will remain fully blinded until Day 70, when a stool sample is taken to assess if participants are shedding the bacterial strains of the vaccine in their faeces. The Investigators and the Monitor will be unblinded to the treatment allocation of participants who have a positive result and the participant will receive a course of antibiotics.

**NUMBER OF PARTICIPANTS:** 45 healthy participants 18 to 45 years of age, inclusive.

**INCLUSION CRITERIA:**

**To be confirmed at Screening**

1. Healthy male and female participants 18 to 45 years of age, inclusive.
2. Female participant of childbearing potential willing to use 2 effective methods of contraception, i.e., established method of contraception + condom, if applicable (unless of non-childbearing potential or where abstaining from sexual intercourse is in line with the preferred and usual lifestyle of the participant) from the first dose until 2 months after the last dose of IMP.
3. Female participant of non-childbearing potential. *For the purposes of this study, this is defined as the participant being amenorrhoeic for at least 12 consecutive months or at least 4 months post-surgical sterilisation (including bilateral fallopian tube ligation or bilateral oophorectomy with or without hysterectomy).*
4. Female participant of childbearing potential or non-childbearing potential with a negative pregnancy test at Screening.
5. Female participant of post-menopausal status confirmed by demonstrating at Screening that the serum level of the follicle stimulating hormone (FSH) falls within the respective pathology reference range. In the event a participant's menopausal status has been clearly established (for example, the participant indicates she has been amenorrhoeic for 10 years, confirmed by medical history, etc), but serum FSH levels are not consistent with a postmenopausal status, determination of the participant's eligibility to be included in the study will be at the Investigator's discretion following consultation with the Sponsor.
6. Male participant willing to use an effective method of contraception or 2 effective methods of contraception, i.e., established method of contraception + condom, if applicable (unless anatomically sterile or where abstaining from sexual intercourse is in line with the preferred and usual lifestyle of the participant) from first dose until a stool sample tested for presence of the vaccine strains is negative.
7. Participant with a body mass index (BMI) of $\geq$ 19 or $\leq$34 kg/m$^2$ (BMI = body weight (kg) / [height (m)]$^2$).
8. No clinically significant history of liver or active gall bladder disease.
9. No clinically significant history of ongoing gastro-intestinal disease or abnormality.
10. No clinically significant history of previous allergy / sensitivity to ZH9/ZH9PA or sodium bicarbonate.
11. No clinically significant history of anaphylactic shock following vaccination.
12. No clinically significant history of hypersensitivity (e.g., hives/rash/swollen lips/difficulty with breathing) to azithromycin, ampicillin, trimethoprim-sulfamethoxazole or ciprofloxacin.
13. No clinically significant abnormal laboratory test results (in the opinion of the investigator) for serum biochemistry, haematology and/or urine analyses within 28 days before receiving the first dose administration of the IMP.
14. Participant with a negative urinary drugs of abuse (DOA) screen (including alcohol and cotinine) test results, determined within 28 days before the first dose administration of the IMP unless there is a documented medical explanation for the positive result other than drugs of abuse (e.g., the

participant has been prescribed opioids for pain). (N.B.: A positive test result may be repeated at the Investigator's discretion).

15. Participant with negative human immunodeficiency virus (HIV), hepatitis B surface antigen (HBsAg)) and hepatitis C virus antibody (HCV Ab) test results at Screening.
16. No clinically significant abnormalities in 12-lead electrocardiogram (ECG) or vital signs determined within 28 days before first dose of IMP.
17. Participant must be available to complete the study (including all follow up visits).
18. Participant must be willing to consent to have data entered into The Over Volunteering Prevention System (TOPS).
19. Participant must provide written informed consent to participate in the study.

**To be re-confirmed on Day 0 / prior to each dosing visit**

1. Participant continues to meet all screening inclusion criteria.
2. Participant with a negative urinary drugs of abuse screen (including alcohol and cotinine) prior to dosing unless there is a documented medical explanation for the positive result other than drugs of abuse (e.g., the participant has been prescribed opioids for pain). (N.B.: A positive test result may be repeated at the Investigator's discretion).
3. Female participant of childbearing potential or non-childbearing potential with a negative pregnancy test on admission.

**EXCLUSION CRITERIA:**

**To be confirmed at Screening:**

1. Participant with any clinically significant medical (cardiovascular disease, pulmonary, hepatic, gallbladder or biliary tract, renal, haematological, gastrointestinal, endocrine, immunologic, dermatological, neurological, autoimmune disease or current infection) or psychiatric condition (see also exclusion criterion number 214) that, in the opinion of the Investigator, precludes participation in the study. This will include any clinically significant abnormal serum biochemistry results and/or haematological results and/or urine analytical results.
2. Participant with a history of heart disease or of rheumatic fever.
3. Participant with a significant acute febrile illness (including fever of 38.0°C or greater within 14 days) of each dose of IMP (Days 0, 21 and 42).
4. Participant who has chronic diseases: Chronic diseases will include all autoimmune and immunocompromising conditions and any other chronic condition, which at the judgment of the Investigator, may put the participant at higher risk of side effects from the study vaccine. Conditions in the latter category might include unexplained anaemia, hepato-biliary disease, uncontrolled hypertension, participant with prosthetic joints or heart valves, etc.
5. Participant with sickle cell anaemia.
6. Participant who has undertaken a course of antibiotics/antibacterials within 28 days prior to each dose of IMP (Days 0, 21 and 42).
7. Use of prescription or non-prescription drugs within 28 days or 5 half-lives (whichever is longer) prior to receiving the first dose of IMP, unless in the opinion of the Investigator and Sponsor's Responsible Physician the medication will not interfere with the study procedures or compromise participant safety.
8. Participant who uses antacids, proton pump inhibitors or $H_2$ blockers on a regular basis or has consumed proton pump inhibitors or $H_2$ blockers within 24 hours prior to each dose of IMP.

9. Participant who has received investigational or licensed vaccines in the 28 days prior to dosing or anticipates receiving a vaccine other than study medication up to Day 84 of the study.
10. Participant with symptoms consistent with Typhoid fever concurrent with travel to countries where typhoid infection is endemic (most of the developing world) within 2 years prior to first dose of IMP.
11. Vaccination against Typhoid within 3 years prior to first dose of IMP.
12. Ingestion of Typhoid bacteria in a challenge study within 3 years prior to dosing.
13. Participant who works as a commercial food handler.
14. Participant who is a health care worker in direct contact with patients.
15. Participant who is a childcare worker.
16. Participant who has household contact with immuno-compromised individuals, pregnant women, children < 2 years of age or individuals > 70 years of age.
17. Participant who has person(s) living with him/her who, in the opinion of the Investigator, may be at risk of disease if exposed to the vaccine strain.
18. Participant with a known impairment of immune function or receiving (or has received in the 6 months prior to study entry) cytotoxic drugs or immunosuppressive therapy (including systemic corticosteroids).
19. Participant who is a current smoker (cigarettes, tobacco and/or e-cigarettes) or has stopped smoking in the last 3 months prior to Screening.
20. A clinically significant history of drug or alcohol abuse [defined as the consumption of more than 14 units of alcohol a week] within the past two years prior to Screening.
21. Inability to communicate well with the Investigators (i.e., language problem, poor mental development or impaired cerebral function).
22. Participation in a New Chemical Entity (NCE) clinical study within the previous 3 months or a marketed drug clinical study within the 30 days before the first dose of IMP. *(Washout period between studies is defined as the period of time elapsed between receiving the last dose of the previous study and receiving the first dose of the next study).*
23. Donation of 450 millilitres (mL) or more blood within the 3 months before the first dose of IMP.
24. Participant who, in the opinion of the Investigator, is unsuitable for participation in the study.

**To be re-confirmed at Day 0 / prior to each dosing visit:**

1. Development of any exclusion criteria since the Screening visit.
2. Use of prescription or non-prescription drugs since Screening, unless in the opinion of the Investigator and Sponsor's Responsible Physician, the medication will not interfere with the study procedures or compromise participant safety.
3. Participation in a clinical study since Screening.
4. Donation of 450 mL or more blood since Screening.

**IMP ADMINISTRATION:**

Cohort 1 will consist of 9 participants, who will receive 3 doses of $1x10^9$ CFU of ZH9PA (6 participants) or placebo (3 participants).

Cohort 2 will consist of 18 participants, who will receive 3 doses of $1x10^{10}$ CFU of ZH9PA (12 participants) or placebo (6 participants).

Cohort 3 will consist of 18 participants, who will receive 3 doses of $1x10^{10}$ CFU of ZH9PA and $1x10^{10}$ CFU of ZH9 (12 participants) or placebo (6 participants).

**CRITERIA FOR EVALUATION:**

Primary Endpoint:

- Local and systemic reactogenicity up to Day 84 as assessed by adverse events, laboratory safety tests (biochemistry, haematology, urinalysis), vital signs and physical examination.

Secondary Endpoints:

- Local and systemic reactogenicity; from Day 85 to Day 224 as assessed by adverse events, laboratory safety tests (biochemistry, haematology, urinalysis) if undertaken for cause; vital signs if undertaken for cause.
- Concentrations of specific serum IgA and IgG antibodies to LPS O:2 and O:9, flagellin H:a and H:d.
- Fold increase in specific serum IgA and IgG antibody concentrations, in individual participants, against the antigens LPS O:2 and O:9, flagellin H:a and H:d.
- Concentrations of specific mucosal IgA ALS assays, in individual participants, against antigens LPS O:2 and O:9, flagellin H:a and H:d.
- Seroconversion rate (proportion of participants with 4-fold increase above baseline at any time post vaccination) against each of the above antigens.
- Fold increase in specific mucosal IgA ALS assays, in individual participants, against the antigens LPS O:2 and O:9, flagellin H:a and H:d.

**STATISTICAL METHODS:** All statistical analysis will be performed using SAS® (version 9.3 or higher).

Descriptive statistics for quantitative parameters will be provided using number of observations (N), mean, standard deviation (SD), minimum, median and maximum. Descriptive statistics for qualitative parameters will be provided using absolute frequencies (n) and relative frequencies (%). For immunological endpoints (IgG, IgA, concentrations and fold increase) additionally geometric means with corresponding 95% confidence intervals (CI) will be presented.

For parameters with evaluation before vaccination and in case of repeated value(s), only the last observation prior to dosing will be used in descriptive and inferential statistics and derivations of other parameter values. After vaccination, only values of scheduled assessments (planned in the protocol) will be used.

The sample size chosen for this study is not based on a formal statistical estimation but is considered to be adequate to meet the objectives of the study. A sufficient number of participants will be initially screened for enrolment to ensure that the planned sample size is achieved.

| | |
|---|---|
| **DURATION OF STUDY:** | Approximately 36 weeks for each individual (from Screening to Post-Study Follow-Up). |

## Table 10.7.1 Study Flow Chart

| | Screening | Treatment Period[1] | | | | | | | | | | | | Post-Study Follow-Up Telephone Call[2] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Week | | 0 | | 1 | 2 | 3 | | 4 | 6 | | 7 | 10 | 12 | 22 | 32 |
| Day | -28 to -1 | 0 | 3 | 7 | 14 | 21 | 24 | 28 | 42 | 45 | 49 | 70 | 84 | 154 | 224 |
| Visit | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| Visit Window | N/A | +/-0 | +/-1 | +/-1 | +/-1 | +/-0 | +/-1 | +/-1 | +/-0 | +/-1 | +/-1 | +/-1 | +/-7 | +/-7 | +/-7 |
| Informed Consent | X | | | | | | | | | | | | | | |
| Inclusion/Exclusion[3] | X | X | | | | X | | | X | | | | | | |
| Demographics | X | | | | | | | | | | | | | | |
| Height / Weight / BMI | X | | | | | | | | | | | | X(weight only) | | |
| Medical History & Concurrent Conditions | X | | | | | | | | | | | | | | |
| Virology Tests | X | | | | | | | | | | | | | | |
| Urine DOA (including Alcohol and Cotinine)[3] | X | X[11] | | | | X[11] | | | X[11] | | | | | | |
| Pregnancy[3, 4] | X(serum) | X(urine) 11 | | | | X(urine) 11 | | | X(urine) 11 | | | | X(serum) | | |
| FSH[5] | X | | | | | | | | | | | | | | |
| Biochemistry[3] | X | X[11] | | X | | X[11] | | X | X[11] | | X | | X | | |
| Haematology[3] | X | X[11] | | X | | X[11] | | X | X[11] | | X | | X | | |
| Urinalysis | X | | X | | | | X | | | X | | | X | | |
| Vital Signs[6] | X | X | X | X | X | X | X | X | X | X | X | | X | | |
| Physical Examination | X | | | | | | | | | | | | X | | |
| General Health Check | | X | | | | X | | | X | | | | | | |
| 12-Lead ECG | X | | | | | | | | | | | | X | | |
| Placebo training | X | | | | | | | | | | | | | | |
| Vaccine administration[7] | | X | | | | X | | | X | | | | | | |

| | Screening | Treatment Period[1] | | | | | | | | | | | Post-Study Follow-Up Telephone Call[2] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Week** | | **0** | | **1** | **2** | **3** | | **4** | **6** | | **7** | **10** | **12** | **22** | **32** |
| **Day** | **-28 to -1** | **0** | **3** | **7** | **14** | **21** | **24** | **28** | **42** | **45** | **49** | **70** | **84** | **154** | **224** |
| **Visit** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** |
| **Visit Window** | **N/A** | **+/-0** | **+/-1** | **+/-1** | **+/-1** | **+/-0** | **+/-1** | **+/-1** | **+/-0** | **+/-1** | **+/-1** | **+/-1** | **+/-7** | **+/-7** | **+/-7** |
| **Twice-Daily Temperature Records[8]** | | ← | → | | | ← | → | | ← | → | | | | | |
| **Blood culture[9]** | | ← | → | | | ← | → | | ← | → | | | | | |
| **Stool bacteriology (vaccine strains)[10]** | | | | | | | | | | | | **X** | **(X)** | | |
| **Administration of antibiotics[10]** | | | | | | | | | | | | **(X)** | | | |
| **Blood sample for serum Ig responses[11]** | | **X** | | | | **X** | | | **X** | | | | **X** | | |
| **PBMC collection for ALS assay[11]** | | **X** | | **X** | | | | **X** | | | **X** | | | | |
| **Adverse Events** | | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** |
| **Concomitant Medication** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** |

**Study Flow Chart Footnotes:**

**All procedures on dosing days (Days 0, 21, 42) are to be completed prior to dosing (except post dosing vital signs and health check for discharge)**

[1] The treatment period will be approximately 85 days duration, from the first dosing day (Day 0) until Day 84 (inclusive).

[2] Post-study follow-up telephone call will be performed on Days 154 and 224 after receiving the first dose of IMP (Day 0).

[3] Eligibility will be confirmed prior to dose administration on Day 0, Day 21 and Day 42.

[4] For female participants only.

[5] For postmenopausal female participants only

[6] At Day 0, Day 21, and Day 42, vital signs to be performed pre-dose and at 4 hours post-dose.

[7] IMP will be administered fasted (90 mins prior to dose administration). Participants will fast for a further 90 mins post each dose administration.

[8] Participants to record body temperature twice daily at home for 7 days post administration of each dose of IMP.

[9] Blood cultures to be performed to identify vaccine strains only if a participant experiences defined symptoms (e.g., fever greater than 37.8°C, white blood cell (WBC) >20x$10^9$ cells/L, respiratory rate >25 breathe per min pulse >130 beats per min (bpm)) or clinically indicated.

[10] Participants will attend the Clinical Unit on Day 70 to provide a stool sample. If the stool sample is positive for the vaccine strains at Day 70, participants will be asked to return to the Clinical Unit to receive a course of antibiotic therapy, and a repeat stool culture will be obtained on Day 84 to confirm shedding of the vaccine strains has ceased.

[11] Samples to be collected prior to each dosing.